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DNA damage and mitochondria dysfunction in cell apoptosis induced by nonthermal air plasma

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Nonthermal plasma is known to induce animal cell death but the mechanism is not yet clear. Here, cellular and biochemical regulation of cell apoptosis is demonstrated for plasma treated cells. Surface type nonthermal air plasma triggered apoptosis of B16F10 mouse melanoma cancer cells causing DNA damage and mitochondria dysfunction. Plasma treatment activated caspase-3, apoptosis executioner. The plasma treated cells also accumulated gamma-H2A.X, marker for DNA double strand breaks, and p53 tumor suppressor gene as a response to DNA damage. Interestingly, cytochrome C was released from mitochondria and its membrane potential was changed significantly. © *2010 American Institute of Physics*. [doi:10.1063/1.3292206]

Despite much effort over the past five decades, we are still having limited success in the war against cancer.[1] As an alternative to conventional methods, physical methods have been proposed in cancer treatment such as nanosecond pulsed electric fields.[2] Nonthermal plasma was also suggested as a selective cancer therapeutic method when coupled with antibody-conjugated nanoparticles.[3]

Plasma research has expanded to include biomedical areas by operating at atmospheric pressure (760 Torr).[4] Atmospheric pressure plasma has the advantages of high density and rich chemical agents without elevation of the substrate temperature. These nonequilibrium characteristics promise applications in the biomedical field, opening a new research area called "Plasma Medicine"[5] which includes sterilization, coagulation, wound healing, and cancer treatment.

Recent research reported that atmospheric pressure plasma can induce apoptosis in mammalian cells.[6] However, the mechanism of apoptosis caused by plasma treatment was not well understood. This letter presents a molecular mechanism of apoptosis induced by nonthermal air plasma.

Figure 1(a) is a schematic diagram of the experimental setup. Surface type air plasma source was developed to treat cancer cells. The plasma source consists of a polytetrafluoroethylene (PTFE) dielectric and Cu electrodes on both sides of the PTFE. The electrode structure was formed by a conventional chemical etching process. The mask pattern for the device and real plasma source are shown in Fig. 1(b).

A high voltage (~15 kV) and low frequency (22 kHz) power supply was connected through the 33 kΩ resistor on the back side. The front side was connected to ground for safety. Figure 1(b) shows the light emission from the plasma operating in ambient air with 4.2 kV (rms) applied voltage. The filamentary discharge was generated consuming 4.26 W and energy density of about 20 J/cm^2 was estimated for the 30 s treatment within the area of the plasma. The temperature remained room temperature (25.5 °C) during the cancer treatment. The plasma source produced lots of ozone (~1000 ppm) which was detected for a long time even when power was turned off.

B16F10 mouse melanoma cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified 5% CO_2 incubator at 37 °C. The cells were loaded on 0.1% gelatin-coated cover glass and grown up to 80% confluence. The cultured cells were treated at 5 mm distance from the plasma source for 10, 30, and 50 s.

Figure 2(a) shows the trypan blue assay upon plasma treatment on B16F10 cells. Before the plasma treatment, the healthy cells, having their ability for selective membrane transport, did not take up trypan blue keeping their morphology [Fig. 2(a), Control]. When the cells were exposed to the plasma, some cells stained with blue color [Fig. 2(a), 0 h] representing perturbed cell membranes. Some stained cells detached from the bottom and floated. As well as the instantaneous effects of plasma on the cell membranes, cellular changes occurred over a longer time period even the cells were placed in fresh media after the plasma treatment. The image denoted as 1 h in Fig. 2(a) shows the trypan blue assay for 1 h incubation after the plasma treatment. Some cells

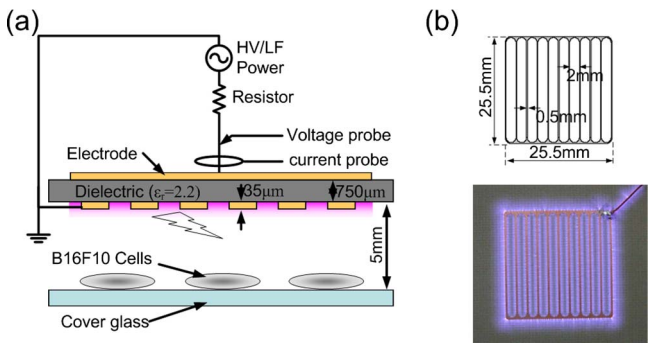

FIG. 1. (Color online) (a) Schematic diagram of experimental setup. (b) Mask pattern for electrode and light emission from the plasma source.

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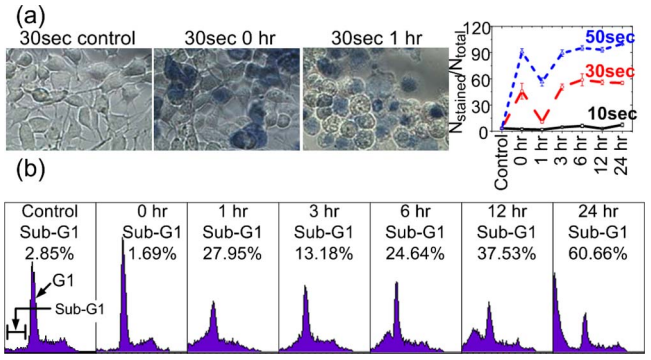

FIG. 2. (Color online) (a) Uptake of trypan blue after plasma treatment to indicate membrane impairment. (b) FACS analysis to check DNA contents in cell populations (treatment for 30 s).

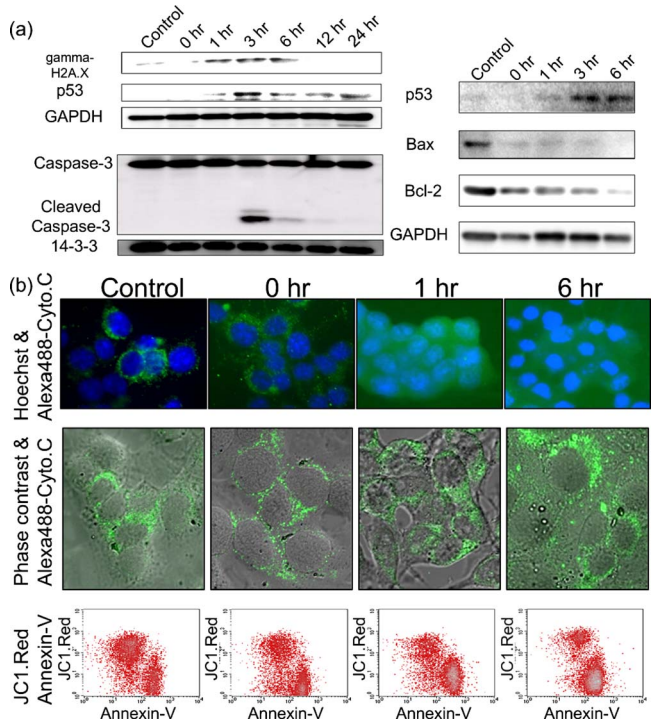

FIG. 3. (Color online) (a) Western blot analysis. (b) The cellular images of cytochrome C release by immunocytochemistry and FACS analysis to check the mitochondria membrane potential change.

were detached from the bottom and changed to round shape but were not stained with trypan blue.

For quantitative data, all harvested cells were loaded to the hemocytometer. The ratio of the number of stained cells ($N_{stained}$) to the total number of cells (N_{total}) was varied with incubation time after the plasma treatment for indicated exposure time [Fig. 2(a)]. There is no significant effect for 10 s treatment. In the case of 30 s, immediately after the plasma irradiation (0 h), 45.8% of cells took up the trypan blue, suggesting membrane damage. Interestingly, the number of stained cells decreased after 1 h incubation showing partial recovery if the damage was not severe. After that, the number of stained cells increased again up to 60% after 3 h, and then the number of stained cells was sustained. Treatment for 50 s shows the similar behavior but increased membrane damage.

DNA contents were also analyzed with the fluorescence-activated cell Sorting (FACS) technique. Figure 2(b) shows the FACS results for DNA contents stained with Propidium iodide (PI) as varying incubation time after the 30 s plasma treatment. Right after the plasma treatment, there was almost no effect on DNA contents (sub-G1 (where G1 represent cell cycle phase that the DNA contents are 2n) of the Control 2.85% and sub-G1 of the plasma treated at 0 h 1.69%). After 1 h following treatment, the sub-G1 population started to appear and gradually increased. This reveals that DNA contents apparently changed upon plasma treatment and it requires a longer time. The peak of sub-G1 phase gradually moved to the less DNA contents region with incubation time, indicating highly fragmented DNA. Finally, at 24 h after plasma treatment, 61% of cells reside in the sub-G1 phase, indicating enriched DNA fragmentation. Hence plasma treatment for short time (30 s) can induce DNA fragmentation. While 10 s treatment showed no effect with that time, 50 s treatment triggered rapid accumulation of sub-G1 population and showed severe DNA damage compared to 30 s treatment.

The possibility of apoptosis was investigated via Western blot analysis following 30 s plasma treatment [Fig. 3(a)]. The cleaved caspase-3 level was clearly increased at 3 h after plasma treatment when compared to 14-3-3 as a loading control, which indicates caspase-associated apoptosis.

To strengthen the possibility that our results were associated with apoptosis, we analyzed a pattern of hH2A.X which is phosphorylated on 139th serine (gamma-H2A.X) when nuclear chromatin contains DNA double strand breaks (DSBs).[7] The increased level of gamma-H2A.X was detected at 3 h after plasma irradiation. The data demonstrate that plasma treatment induced DNA damage such as DSBs.

Cells have defense mechanisms against internal disturbance of genomic integrity or external stresses. When the defense systems are operating well, the cells recover and resume their function. However, if the damage is too severe, the recovery process is withdrawn and, in turn, the apoptosis process is initiated to prevent the proliferation of cells with damaged DNA. The p53 tumor suppressor gene is a typical example of molecular sensor in response to DNA damage.[8] Air plasma treatment induced accumulation of the p53 in response to DNA damage.

In determining whether a cell will live or die, BCL-2 family members have a role. The BCL-2 family proteins are classified as proapoptotic (Bax, Bak, etc.) and antiapoptotic (Bcl-2, Bcl-X_L, etc.). The fate of a cell depends partially on the ratio of these two subgroups.[9] After the plasma irradiation, the total levels of pro-(Bax) and anti-(Bcl-2) apoptotic protein were also analyzed. Bcl-2 decreased gradually with incubation time after the plasma irradiation, supporting the activated caspase cascade. However, Bax also decreased after the plasma treatment. This is a difference between plasma treatment and usual Bax dependent cytochrome C release. In our experimental conditions, the ratio between antiapoptotic protein (Bcl-2) and proapoptotic protein (Bax) was not correlated with known cytochrome C release.

We further analyzed cellular location of cytochrome C with the immunocytochemistry [Fig. 3(b)]. In the case of control, cytochrome C was localized in the mitochondria represented by green spots. Cytochrome C was released from the mitochondria to the cytosol at 1 h as shown smeared green fluorescent and gradually released at later times. The results suggest that plasma treatment can damage to cellular

organelles such as mitochondria. With these results, we suggest that the effects of plasma on cells involve genetic signaling cascade from DNA-damage and simple nongenetic physical damage in cellular membrane systems.

For further considerations, we next analyzed mitochondrial membrane potential by using JC1, mitochondrial membrane-specific fluorescent dye[10] [Fig. 3(b)] In this assay, we discovered that nonapoptotic and JC1-red positive cell population were greatly reduced immediately after plasma treatment. This population was slightly recovered in 1 h, but never replenished significantly after 3 h. This pattern is in accord with trypan blue assay. Our results present that mitochondrial membrane potential was greatly perturbed immediately after plasma treatment. Then small population tried to recover it but never returned. On the other hands, pattern of Annexin-V, marker for apoptotic progression, was in accord with PI contents of plasma treated cells as previously reported.[6]

In summary, we have demonstrated DNA damage, mitochondria dysfunction, and markers that indicate caspase-associated apoptosis in B16F10 cells induced by nonthermal air plasma. The surface type nonthermal air plasma operated in ambient air generates a lot of ozone, which could be responsible for DNA damage. Plasma-induced cell apoptosis was obviously identified by not only the increased sub-G1 population but also activated caspase-3. The plasma treated cells experienced DNA damage such as DSBs and were mediated by a p53 dependent signaling cascade. Interestingly, while both anti-(Bcl-2) and pro-(Bax) apoptotic proteins were decreased, cytochrome C was released after plasma treatment. According to JC1 assay, we observed that mitochondrial membrane potential was changed due to the plasma treatment.

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